RESEARCH COMMUNICATION

HSP43, a small heat-shock protein localized to specific cells of the vulva and spermatheca in the nematode *Caenorhabditis elegans*

Lily DING and E. Peter M. CANDIDO[1]

Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada V6T 1Z3

Heat-shock protein 43 (HSP43) of *Caenorhabditis elegans* is prominently expressed in the utse cell, which attaches the uterus to the hypodermis, the uv1 cells joining the vulva and the uterus, the spermathecal valve and junctions between cells of the spermathecal cage. In body-wall muscle, HSP43 forms a punctate pattern of circumferential lines, probably corresponding to regions where the hypodermis contacts the muscle cells.

Key words: α-crystallin, immunocytochemistry, reproductive organs, stress protein, tissue specificity.

INTRODUCTION

Small heat-shock proteins (smHSPs) belong to the α-crystallin family [1–3]. Whereas the subunit molecular masses of these proteins range from approx. 12000 to 43000, they generally exist as large complexes in their native forms. The best characterized smHSP structure, that of HSP16.5 from the thermophilic archaeon *Methanococcus jannaschii*, consists of a spherical assembly of 24 subunits [4]. Some smHSPs are strictly stress-inducible, whereas others may be constitutively expressed, developmentally regulated or up-regulated under stress. Although the functions of smHSPs *in vivo* are poorly understood, they show an ATP-independent chaperone activity *in vitro*, i.e. the ability to inhibit aggregation and precipitation of substrate proteins [5]. SmHSPs have been found to be associated with specific structures in some cells, such as intermediate filaments [6], adherens junctions of desmosomes [7] or co-localized with muscle components [7,8]. A mixed complex between the small heat-shock proteins myotonic dystrophy protein kinase-binding protein/HSPB2 and HSPB3 is formed during muscle differentiation in rat [8]. These findings suggest that smHsps may serve a variety of specific functions in different tissues and cell types. Here we report the characterization of a new smHSP, HSP43, the largest identified member of this protein family encoded in the genome of the nematode *Caenorhabditis elegans*. HSP43 is constitutively expressed, developmentally regulated and shows striking localization to specific somatic cells of the vulva and spermatheca, and to body-wall-muscle cell contacts.

MATERIALS AND METHODS

Cloning and expression of HSP43

To generate the expression vector pRSET43, the HSP43 cDNA was amplified from phagemid pRATII-CM14F11 (a gift from Dr Chris Martin, Genome Sequencing Center, Washington University, St. Louis, MO, U.S.A.) with primers LD1 (5′ tga catATG ACT CTT GCA ACC CGT CAT 3′, forward primer) and LD2 (5′ cga ctgcag TTA ATA TGT CTT GCG GAG 3′, reverse primer). The amplified HSP43 sequence (nucleotides 1–1107 of the coding region) was cloned into the *Nde*I–*Pst*I sites of pRSET (Invitrogen). The vector pET43H$_6$ encoding HSP43 with a C-terminal His$_6$ tag (HSP43H$_6$) was prepared by amplifying and subcloning the HSP43 coding region from pRSET43 into the *Nco*I–*Hin*dIII sites of pET28a(+) (Novagen) using primers LD20 (5′ aat ccatgg gc ACT CTT GCA ACC CGT CAT 3′, forward primer) and LD14 (5′ ccgaagctt ATA TGT CTT GCG GAG AAT 3′, reverse primer). HSP43H$_6$ was produced in *Escherichia coli* BL21(DE3) cells as described previously [7]. Bacterial cell pellets were suspended in 20 ml of Denaturing Buffer (6 M guanidinium chloride/50 mM Tris, pH 7.5), centrifuged at 12000 *g* for 10 min, and the supernatant was loaded onto a Ni^{2+}-nitrilotriacetate–agarose column (Qiagen; 1 cm × 6.5 cm; 5 ml bed volume). The column was washed with 50 ml of Denaturing Buffer containing 10% (v/v) glycerol and 1% (v/v) Triton X-100, followed by 10 ml of Denaturing Buffer, and eluted with 0.5 M imidazole in Denaturing Buffer. After dialysis against PBS (137 mM NaCl/2.7 mM KCl/4.3 mM Na$_2$HPO$_4$·7H$_2$O/1.4 mM KH$_2$PO$_4$, pH 7.4), HSP43H$_6$ precipitated from solution. The insoluble protein was collected by centrifugation, and an aliquot was analysed by SDS/PAGE to confirm purity [9]. The HSP43H$_6$ was then used for antibody production as described in [7].

Developmental expression profile

Western blots of proteins from control and heat-shocked wild-type *C. elegans* were carried out as described in [7]. Immobilon-P membranes were probed with rabbit anti-HSP43 antibody {1:10000 dilution, pretreated with 1% (w/v) *E. coli* acetone-dried powder [10]} or with an anti-actin monoclonal antibody (Clone 4, 1:10000 dilution; ICN), followed by the appropriate secondary antibody [horseradish peroxidase-conjugated donkey anti-rabbit secondary antibody, 1:10000 dilution (Amersham) or horseradish peroxidase-conjugated mouse anti-rabbit secondary antibody, 1:10000 dilution (Promega)]. Protein–antibody complexes were detected by enhanced chemiluminescence (ECL®; Amersham). For comparison of different developmental stages, samples were adjusted so as to yield approximately equal signals with the anti-actin antibody.

Size-exclusion column chromatography

Nematode extract (100 μl) was loaded onto a Superdex 200 column (24 ml bed volume; Amersham Pharmacia Biotech) and chromatographed on an AKTA system (Amersham Pharmacia Biotech) at a flow rate of 0.7 ml/min. Fractions (0.25 ml each) were collected, and aliquots were analysed by SDS/PAGE, followed by Western blotting with the anti-HSP43 antibody.

Immunocytochemistry

Mixed populations of N2 nematodes were cultured at 20 °C [11] or heat-shocked at 33 °C as described previously [12]. Immuno-fluorescence staining was a modification of the method of Loer and Kenyon [13], as previously described [7]. After blocking in BSA at room temperature for 1 h, permeabilized nematodes were incubated with rabbit anti-HSP43 antibody (pre-treated with 1% *E. coli* acetone-dried powder) at 1:250 dilution, and/or mouse monoclonal antibodies MH27 [14] or DM5.6 [16] at 1:250 dilution at room temperature for 2 h. Samples were washed and incubated with fluorescent secondary antibodies at 1:250 dilution (Alexa™488 anti-rabbit conjugate from Molecular Probes, Inc. and/or Texas Red dye-conjugated anti-mouse IgG from Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.) at room temperature for 2 h. In combination with antibody staining, some nematodes were also stained with 4,6-diamidino-2-phenylindole (DAPI) and Texas Red-X–phalloidin (Molecular Probes). In control experiments, anti-HSP43 antibody was pre-incubated with 1 mg/ml of recombinant HSP43H$_6$ prior to use. After staining, nematodes were mounted as described in [7] and viewed by fluorescence microscopy on an Axioplan 2 microscope (Zeiss).

Double-stranded-RNA-mediated interference (RNAi) assays of HSP43

Double-stranded RNA was prepared from phagemid pRATII-CM14F11 (nucleotides 1–1107 of the HSP43 coding region) or cDNA clone YK275 (phagemid pBluescript SK−; nucleotides 117–1107 of the HSP43 coding region; a gift from Dr. Yuji Kohara, National Institute of Genetics, Mishima, Japan). Double-stranded RNA was synthesized as described by Fire et al. [17] and microinjected into nematodes as previously described [7].

RESULTS AND DISCUSSION

Polyclonal antibodies produced to recombinant HSP43 reacted with a single major protein species with an apparent molecular mass of 43 000, and was present at similar levels in both control and heat-shocked animals (Figure 1A). To investigate the developmental expression of HSP43, nematode extracts were prepared from all life-cycle stages and probed with anti-HSP43 antibody (Figure 1B). HSP43 was detected at all developmental stages in animals growing at normal temperature, although levels in embryos were somewhat lower. HSP43 was surprisingly unstable in nematode extracts (even when stored on ice in SDS loading buffer, with protease inhibitors), levels becoming almost undetectable within 15 min. In contrast, actin was stable under the same conditions (Figure 1C). Given this finding, quantitative comparisons of HSP43 levels at different developmental stages must be interpreted with caution, since it is possible that the half-life of HSP43 in extracts could vary at different stages.

We attempted to estimate the size of the HSP43 complex in fresh nematode extracts by size-exclusion chromatography. HSP43 was eluted in the void volume from a column of Superdex
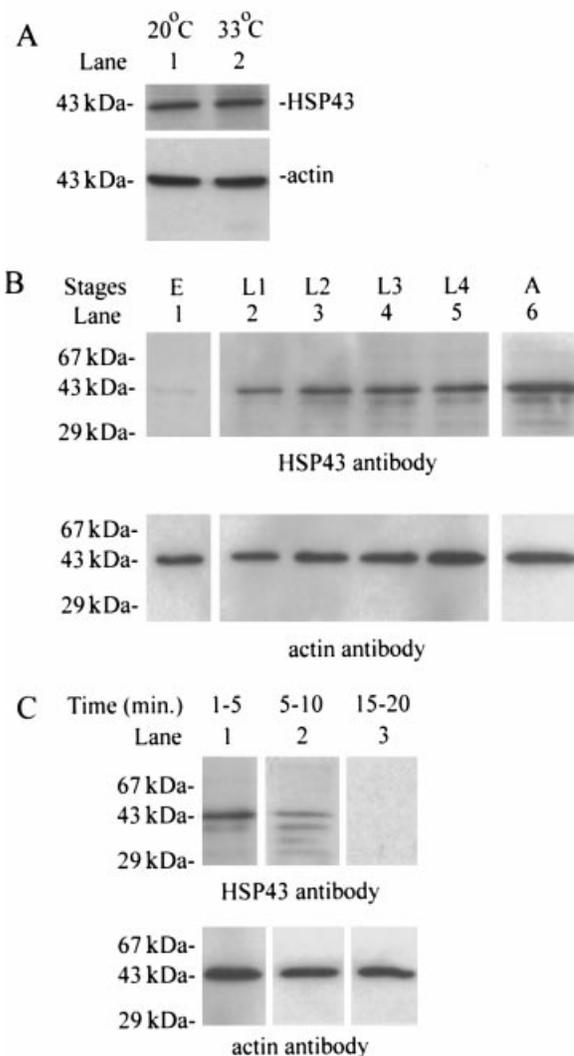
Figure 1 Expression of HSP43

(**A**) Effect of heat shock on HSP43 synthesis. *C. elegans* was cultured either at 20 °C (lane 1) or heat-shocked at 33 °C (lane 2). Extracts were analysed by SDS/PAGE and Western blotting (upper panel, antibody to HSP43; lower panel, antibody to actin). (**B**) Extracts were prepared from *C. elegans* at each developmental stage and analysed by SDS/PAGE and Western blotting as in (**A**). Lane 1, embryo; lanes 2–5 (L1–L4 larval stages); lane 6, adult. (**C**) Degradation of HSP43. Fresh nematode extracts in 1 × SDS/PAGE loading buffer were treated as described in the Materials and methods section and incubated on ice for the indicated times before SDS/PAGE analysis.

200, as shown by Western blotting of column fractions with the HSP43 antibody. The size of the complex was estimated to be more than 670 kDa (results not shown), suggesting that HSP43 forms multimers of at least 16 subunits.

The tissue specificity of HSP43 in larval and adult nematodes was examined by immunofluorescence staining. In both control and heat-shocked adult and L4 larvae (the fourth larval stage) a low level of staining was seen throughout the body, together with very strong, specific, staining in the vulva and spermatheca (Figure 2A). In the vulval region, HSP43 was localized in the adherens junctions of vulval epithelial cells (Figure 2B), but absent from vulval muscle (Figure 2C). Images taken in different focal planes of the vulval region revealed HSP43 staining in the utse cells, which attach the uterus to the seam cells of the
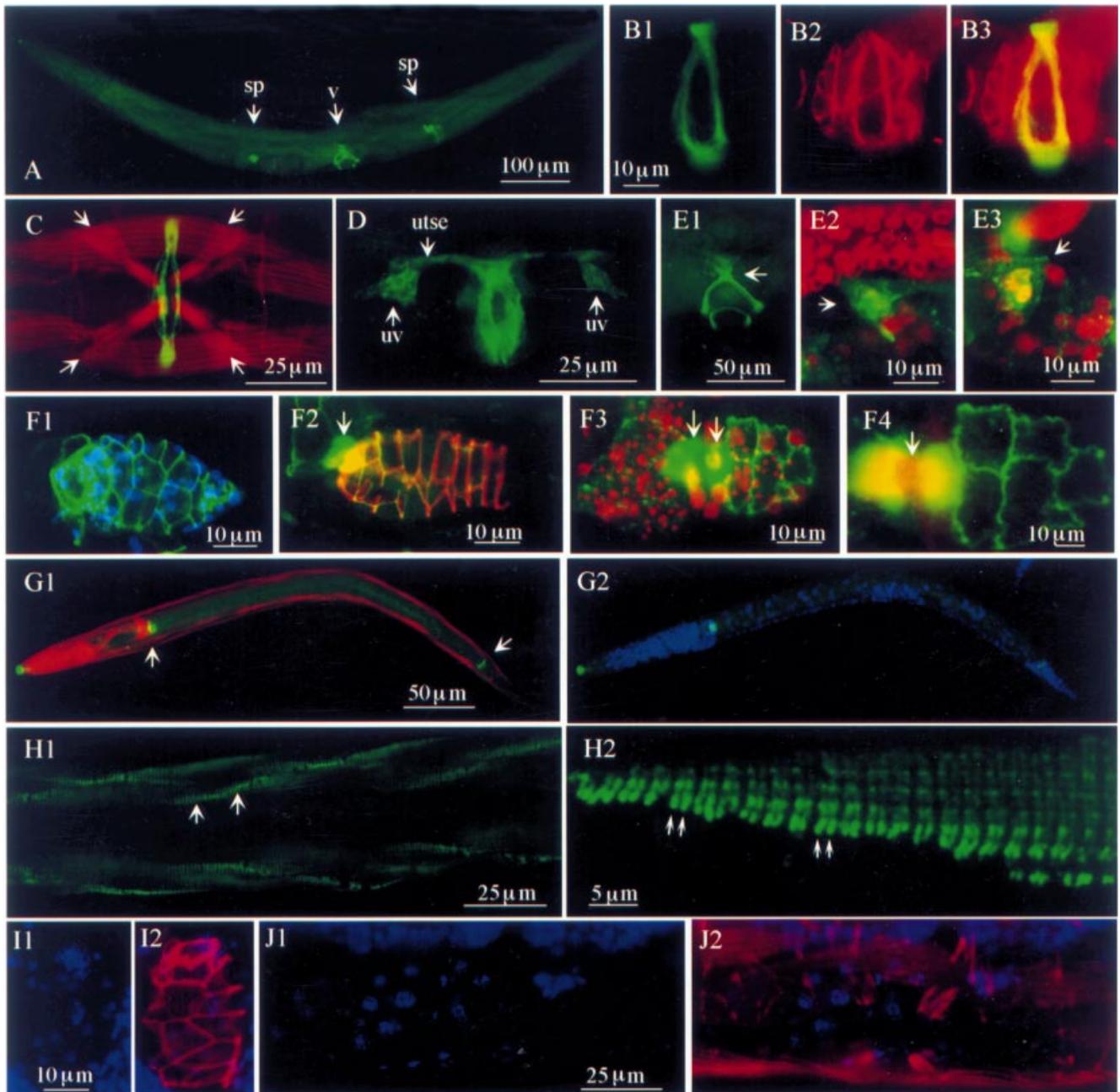
Figure 2   Immunocytochemistry of HSP43

All animals orientated with anterior to the left unless otherwise mentioned. In all images, HSP43 staining is shown in green. (**A**) Young adult. HSP43 is concentrated in the vulva (v) and spermathecae (sp). (**B**) HSP43 expression in the adherens junctions of vulval epithelia (ventral view). HSP43 (**B1**) and MH27, which stains desmosomes (**B2**), are partially co-localized (**B3**). (**C**) Ventral view. The relative position of HSP43 in vulva (green) is compared to vulval muscle (red, arrows, phalloidin). (**D**) Overview of HSP43 staining in vulval region (adult, lateral view). Arrows indicate utse and 2 uv1 cells. (**E1**) H-shaped utse, ventro-lateral view. (**E2**) and (**E3**) uv1 cells, with adjacent DAPI-stained gonadal nuclei (shown in red). (**F**) Spermathecal expression of HSP43. (**F1**) HSP43 staining in the spermathecal cage (green) with DAPI-stained sperm nuclei (shown in blue). (**F2**) HSP43 (green) is co-localized with desmosomes (red, MH27), except in spermathecal valve (arrow). (**F3**) More lateral view of a spermathecal cage, with HSP43 staining in green and nuclei (DAPI) in red. (**F4**) HSP43 staining in spermathecal valve region (green) compared with valve-muscle staining (red, phalloidin); HSP43 labels both ends of the phalloidin pattern. (**G1**) and (**G2**) L2 showing staining of muscle in red (phalloidin, **G1**) and nuclei in blue (DAPI, **G2**). The pharyngeal–intestinal valve (left arrow, **G1**) and intestinal–anal valve (right arrow, **G1**) are labelled by HSP43. (**H**), HSP43 staining over body-wall muscle. (**H1**) L3 larva. The signal is more intense where muscle cells contact one another (arrows). (**H2**) magnified region of L3-larval muscle surface, showing punctate pattern of HSP43 staining forming columns running circumferentially over muscle cells, probably at sites of hypodermal contacts. Columns can be resolved as doublets of elongated spots (arrows). (**I**) and (**J**) Specificity of anti-HSP43. No HSP43 signal was seen in spermatheca (**I**) or vulva (**J**) when antiserum was pre-incubated with excess pure HSP43. DAPI staining (blue) shows location of nuclei (**I1** and **J1**), and co-staining with MH27 (red, **I2**) or DM5.6 (red, **J2**) indicates efficient permeabilization of the animals.

hypodermis (Figures 2D and 2E1) and in the uv1 cells which form the junction between the vulva and uterus [18] (Figures 2D, 2E2 and 2E3). At the same developmental stages, HSP43 staining was seen in desmosomes of cells which make up the spermathecal cage (Figure 2, F1–F4) and the spermathecal valve (Figure 2, F2–F4). L2 larvae, which lack vulva and spermatheca, showed prominent HSP43 staining anteriorly in the pharyngeal–intestinal valve, a structure made up of six cells coupled by a system of desmosomes [19], and posteriorly in a discreet region probably corresponding to the intestinal–rectal valve (Figure 2, G1 and G2).

In all post-embryonic stages, HSP43 staining was observed in a very regular punctate pattern over body-wall muscle, the columns of labelled spots running circumferentially along the muscle quadrant and the signal being more intense where the muscle cells contact each other (Figure 2, H1, arrows). Individual bands could be resolved as doublets separated by a narrow space (Figure 2, H2, arrows). This pattern appears identical with that reported for monoclonal antibody MH5 [20], which labels fibrous organelles containing hemidesmosomes associated with the hypodermis, where it contacts the muscle cells. Similar patterns, but differing in some details, were noted with monoclonal antibodies MH4 and MH46 [20]. Interestingly, the monoclonal antibody IFA, which defines an epitope conserved on all classes of mammalian intermediate filaments [21], also reacts with arrays of filaments in *C. elegans* [20] and *Ascaris* [22], and yields an immunofluorescence pattern very similar to those of MH4 and MH5 [20]. The specificity of the antiserum for HSP43 is demonstrated by the loss of signal when the antibody is pre-incubated with excess pure recombinant HSP43 (Figure 2, I1–J2).

Recently a number of associations between smHSPs and cytoskeletal elements have been noted. α-Crystallins have been implicated in modulating intermediate-filament assembly [23] and actin polymerization [24], and HSP27 is associated with glial fibrillary acidic protein (a class of intermediate filament), vimentin and keratin filaments in various mammalian cell lines [6]. Characterization of five of the six known mammalian smHSPs revealed that only muscle-related tissues expressed all types [8]. Our recent studies of HSP25, another *C. elegans* smHSP, showed it to be localized to dense bodies and M lines in body muscle, and to junctions between cells of the spermathecal wall [7]. The latter resembled the pattern seen here with HSP43. Thus an increasing body of evidence implicates the smHSPs in managing interactions between protein components of filamentous networks in cells.

To examine the possible phenotype of an HSP43 knock-out mutation, we carried out RNAi experiments. This technique, which involves micro-injection of double-stranded RNA corresponding to a given gene sequence, results in potent and specific inhibition of gene expression in the F1 progeny of the injected animal, and often mimics the null phenotype of the gene [17,25]. Under conditions which produced suppression of a control myosin–green fluorescent protein fusion gene in approx. 90% of the progeny of injected animals, no effect of HSP43 RNAi on embryo viability was seen, and the progeny of the injected nematodes developed normally (results not shown). The lack of a conspicuous phenotype in these RNAi experiments suggests, but does not prove, that the function of HSP43 is non-essential in normal *C. elegans* growth and development. Other possibilities are that up-regulation of another member of the smHSP family might be able to compensate for a decrease in

HSP43, or that HSP43 might be essential only under specific physiological conditions. It is unlikely that RNAi experiments would reveal a moderate selective advantage conferred by HSP43 function, which could be of great significance under natural conditions.

We have shown that HSP43 expression shows remarkable tissue specificity in *C. elegans*, being localized prominently to the utse and uv1 cells of the vulva, the somatic cells forming the spermatheca and the spermathecal valve. HSP43 is also found at sites of contact between body-wall muscle cells and, tentatively, between muscle cells and overlying hypodermis. In the light of demonstrated associations between smHsps and intermediate filaments [6,23], one might envisage, for instance, that HSP43 is particularly well adapted for chaperoning intermediate-filament assembly ↔ disassembly in the above tissues.

REFERENCES

1 Gething, M.-J. (ed.) (1997) Guidebook to Molecular Chaperones and Protein-Folding Catalysts, pp. 269–290, Oxford University Press, New York

2 de Jong, W. W., Caspers, G.-J. and Leunissen, J. A. M. (1998) Int. J. Biol. Macromol. **22**, 151–162

3 Ehrnsperger, M., Buchner, J. and Gaestel, M. (1997) in Molecular Chaperones in the Life Cycle of Proteins (Fink, A. L. and Goto, Y., eds.), pp. 533–575, Marcel Dekker, New York

4 Kim, K. K., Kim, R. and Kim, S.-H. (1998) Nature (London) **394**, 595–599

5 Horwitz, J. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 10449–10453

6 Perng, M. D., Cairns, L., van den IJssel, P., Prescott, A., Hutcheson, A. M. and Quinlan, R. A. (1999) J. Cell Sci. **112**, 2099–2112

7 Ding, L. and Candido, E. P. M. (2000) J. Biol. Chem. **275**, 9510–9517

8 Sugiyama, Y., Suzuki, A., Kishikawa, M., Akutsu, R., Hirose, T., Waye, M. M. Y., Tsui, S. K. W., Yoshida, S. and Ohno, S. (2000) J. Biol. Chem. **275**, 1095–1104

9 Laemmli, U. K. (1970) Nature (London) **227**, 680–685

10 Harlow, E. and Lane, D. (1988) Antibodies: A Laboratory Manual, p. 633, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

11 Brenner, S. (1974) Genetics **77**, 71–94

12 Stringham, E. G., Jones, D. and Candido, E. P. M. (1992) Mol. Biol. Cell **3**, 221–233

13 Loer, C. M. and Kenyon, C. J. (1993) J. Neurosci. **13**, 5407–5417

14 Francis, G. R. and Waterston, R. H. (1985) J. Cell Biol. **101**, 1532–1549

15 Reference deleted

16 Miller, III, D. M., Ortiz, I., Berliner, G. C. and Epstein, H. F. (1983) Cell **34**, 477–490

17 Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C. (1998) Nature (London) **391**, 806–811

18 Sharma-Kishore, R., White, J. G., Southgate, E. and Podbilewicz, B. (1999) Development **126**, 691–699

19 White, J. (1988) in The Nematode *Caenorhabditis Elegans* (Wood, W. B., ed.), pp. 103–105, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

20 Francis, R. and Waterston, R. H. (1991) J. Cell Biol. **114**, 465–479

21 Pruss, R. M., Mirsky, R., Raff, M. C., Thorpe, R., Dowding, A. J. and Anderton, B. H. (1981) Cell **27**, 419–428

22 Bartnik, E., Osborn, M. and Weber, K (1986) J. Cell Biol. **102**, 203–2041

23 Nicholl, I. D. and Quinlan, R. A. (1994) EMBO J. **13**, 945–953

24 Arrigo, A.-P. and Landry, J. (1994) in The Biology of Heat Shock Proteins and Molecular Chaperones (Morimoto, R. I., Tissières, A. and Georgopoulos, C., eds.), pp. 335–373, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

25 Fire, A. (1999) Trends Genet. **15**, 358–363